JOURNAL OF VIROLOGY, Jan. 2009, p. 29–36 Vol. 83, No. 1
0022-538X/09/$08.00+0 doi:10.1128/JVI.02293-07

Interaction of the Influenza A Virus Nucleocapsid Protein with the Viral RNA Polymerase Potentiates Unprimed Viral RNA Replication▽

Laura L. Newcomb,[1]†‡ Rei-Lin Kuo,[1]‡ Qiaozhen Ye,[2] Yunyun Jiang,[2] Yizhi Jane Tao,[2] and Robert M. Krug[1]*

Institute for Cellular and Molecular Biology, Section of Molecular Genetics and Microbiology, University of Texas at Austin, Austin, Texas 78712,[1] and Department of Biochemistry and Cell Biology, Rice University, Houston, Texas 77005[2]

Received 22 October 2007/Accepted 10 October 2008

The influenza A virus polymerase transcribes and replicates the eight virion RNA (vRNA) segments. Transcription is initiated with capped RNA primers excised from cellular pre-mRNAs by the intrinsic endonuclease of the viral polymerase. Viral RNA replication occurs in two steps: first a full-length copy of vRNA is made, termed cRNA, and then this cRNA is copied to produce vRNA. The synthesis of cRNAs and vRNAs is initiated without a primer, in contrast to the initiation of viral mRNA synthesis, and requires the viral nucleocapsid protein (NP). The mechanism of unprimed viral RNA replication is poorly understood. To elucidate this mechanism, we used purified recombinant influenza virus polymerase complexes and NP to establish an in vitro system that catalyzes the unprimed synthesis of cRNA and vRNA using 50-nucleotide-long RNA templates. The purified viral polymerase and NP are sufficient for catalyzing this RNA synthesis without a primer, suggesting that host cell factors are not required. We used this purified in vitro replication system to demonstrate that the RNA-binding activity of NP is not required for the unprimed synthesis of cRNA and vRNA. This result rules out two models that postulate that the RNA-binding activity of NP mediates the switch from capped RNA-primed transcription to unprimed viral RNA replication. Because we showed that NP lacking RNA-binding activity binds directly to the viral polymerase, it is likely that a direct interaction between NP and the viral polymerase results in a modification of the polymerase in favor of unprimed initiation.

Influenza A viruses cause a contagious respiratory disease in humans and are responsible for periodic, high-mortality pandemics (45). The 1918 pandemic resulted in approximately 30 million deaths worldwide (36), and the avian H5N1 viruses, which have spread from Asia to Europe and Africa, are highly virulent, resulting in death in approximately 65% of infected humans (44). It is not known why these viruses are virulent, and only a few of the molecular determinants of their virulence have been identified (10, 27). The viral RNA-dependent RNA polymerase, which is comprised of three proteins (PA, PB1, and PB2), has been implicated in the virulence of the 1918 and H5N1 viruses, but the underlying molecular mechanisms have not been elucidated (9, 11, 38). For example, H5N1 viruses that are virulent in mice contain lysine at position 627 in the PB2 protein, whereas H5N1 viruses that are not virulent in mice, as well as other avian influenza A viruses, contain glutamic acid at this position (9). This amino acid change may represent an adaptation for efficient replication in mammals (5, 40, 42), although this change is not required for virulence in ferrets (48). In addition, amino acids in PB1 and PA have been implicated in virulence in ferrets and/or ducks (11, 38). It has been postulated that influenza virus RNA synthesis requires host factors that differ between species (5, 17, 25, 40, 42), and evidence for the participation of host factors in influenza virus RNA synthesis has been reported (12, 13, 21, 23, 24, 26, 39).

These results highlight the importance of elucidating the molecular mechanisms of the influenza virus RNA polymerase, which functions in the nucleus to both transcribe and replicate the eight segments of virion RNA (vRNA) (15). The polymerase is associated with each of the vRNA segments, which contain nucleocapsid (NP) protein molecules bound at 24-nucleotide (nt) intervals along their entire length, thereby forming viral ribonucleoproteins (RNPs) (1, 4, 8). Transcription is initiated with a 10- to 13-base-long capped RNA primer excised from cellular pre-mRNAs by the intrinsic endonuclease of the viral polymerase (15, 35). This cap-dependent endonuclease, which is activated by the binding of the common 5′ terminus of vRNA to a specific amino acid sequence in the PB1, is comprised of a cap binding site on the PB2 protein (which does not include amino acid 627) and an endonuclease active site on the PB1 protein (18, 19). The common 3′ end of the vRNA binds to another site on the PB1 subunit (18), and the vRNA is copied until the polymerase reaches a short (4- to 7-base) U sequence 15 to 22 bases from the vRNA 5′ end, at which point poly(A) is added by reiterative copying of the U tract followed by termination (15, 32, 37). Consequently, the resulting viral mRNAs are not complete copies of the vRNAs.

Less is known about the mechanism of viral RNA replication. Viral RNA replication occurs in two steps: first a full-length copy of the vRNA is made, termed cRNA, and then this cRNA is copied to produce vRNA. The synthesis of cRNAs and vRNAs is initiated without a primer, in contrast to the initiation of viral mRNA synthesis, and requires the viral NP protein (15). One role of NP during viral RNA replication is to

* Corresponding author. Mailing address: Institute for Cellular and Molecular Biology, University of Texas at Austin, 2500 Speedway, Austin, TX 78712. Phone: (512) 232-5563. Fax: (512) 232-5565. E-mail: rkrug@mail.utexas.edu.

† Present address: Biology Department, California State University San Bernardino, San Bernardino, CA 92407.

‡ These two authors made equal contributions to this work.

▽ Published ahead of print on 22 October 2008.

block termination at the U tract, thereby enabling the polymerase to synthesize cRNA, a complete copy of the vRNA template (2). Three models for other roles of NP in the switch from capped RNA-primed transcription to unprimed replication have been proposed (33, 43). Two of these models attribute the switch to the RNA-binding activity of NP. In one model, the role of NP is to stabilize nascent cRNA and vRNA transcripts that would otherwise be degraded in the absence of NP, designated as the "stabilization model" (43). In the second model, NP binds to the template RNA and alters its structure to favor unprimed RNA synthesis, designated as the "template modification model" (33). The third model proposes a direct protein-protein interaction between NP and one or more subunits of the viral polymerase, resulting in a modification of the polymerase in favor of unprimed initiation, designated as the "polymerase modification model" (33).

Our approach to elucidating the molecular mechanisms of influenza virus RNA replication was to establish an in vitro system using purified recombinant proteins. Specifically, the PA, PB1, and PB2 proteins were coexpressed in insect cells using baculovirus vectors, resulting in the formation of polymerase complexes that were then purified. The NP protein was separately expressed using a baculovirus vector and purified. We used 50-nt-long vRNA and cRNA as templates for the synthesis of cRNA and vRNA, respectively. We show that the purified recombinant polymerase complexes synthesize 50-nt-long cRNA and vRNA in the absence of a primer when supplemented with purified recombinant NP protein, suggesting that host cell factors are not required for unprimed initiation of viral RNA replication. Further, we utilize this purified system to show that the RNA-binding activity of NP is not required for the stimulation of unprimed viral RNA replication and that NP lacking RNA-binding activity binds directly to the viral polymerase. These results show that the "polymerase modification model" is most likely the mechanism by which NP mediates the switch from transcription to viral RNA replication.

MATERIALS AND METHODS

Expression and purification of the influenza virus polymerase and NP. All recombinant proteins were produced using the Bac-to-Bac baculovirus expression system. Sf21 insect cells were grown in Hink's TNM-FH insect medium supplemented with 10% fetal bovine serum. To prepare the purified trimeric influenza virus polymerase containing PA, PB1, and PB2 proteins, Sf21 cells were infected with three recombinant baculoviruses encoding PA with an N-terminal His tag and nontagged PB1 and PB2 proteins of influenza A/PR/8/34 virus. Cells were harvested 64 h postinfection, and the cell pellet was resuspended in a lysis buffer (50 mM Tris-HCl, pH 8.0; 300 mM NaCl; 5 mM imidazole; 10% glycerol; 0.2 mM 2-mercaptoethanol; 1 μg/ml pepstatin; 1 μg/ml leupeptin; 17 μg/ml phenylmethylsulfonyl fluoride; 20 μg/ml DNase I; 100 μg/ml RNase A), and cells were disrupted with a microprobe sonicator. After centrifugation at 25,000 × *g* for 20 min at 4°C to remove cell debris, the supernatant was mixed with Qiagen nickel-nitrilotriacetic acid (Ni-NTA) agarose resin for 1 h, which was washed twice with buffer containing 20 mM imidazole, followed by elution of bound proteins with buffer containing 250 mM imidazole. The eluted proteins were applied to a 1-ml HiTrap Heparin HP column (Amersham Biosciences). The trimeric viral polymerase was eluted from this column at NaCl concentrations between 500 mM and 900 mM. The peak polymerase fractions were further purified by gel filtration chromatography on a Superose 6 column in buffer A (50 mM Tris-HCl, pH 7.6; 200 mM NaCl; 1 mM EDTA; 10% glycerol; 0.2 mM 2-mercaptoethanol). The purified trimeric viral polymerase, which was eluted at a molecular mass of ~250 kDa, was used for the experiments described here. Both untagged and C-terminally His-tagged NP (C-His-NP) were purified. Sf21 cells were infected with a baculovirus encoding either untagged or C-His-NP protein from influenza A/WSN/33 virus. The cells were harvested 48 h postinfection, the cell pellet was resuspended in the lysis buffer, and cells were disrupted by sonication and clarified by centrifugation as described above. For the purification of untagged NP, the supernatant was mixed with 30% (wt/vol) ammonium sulfate for 1 h at 4°C before centrifugation (30,000 × *g*, 30 min, 4°C). The salted-out proteins were dissolved in buffer A and dialyzed against buffer A containing 100 mM NaCl for 3 h at 4°C. After centrifugation to remove insoluble material, the supernatant was applied to a 5-ml HiTrap Heparin HP column (Amersham Biosciences). The NP protein was eluted from this column at an NaCl concentration of 900 mM. The NP protein peak was collected and purified by gel filtration using a Superdex 200 or Superose 6 column in buffer A containing 200 mM NaCl. The final purification step used a Mono S HR 5/5 column that was eluted with a linear salt gradient (100 mM to 600 mM NaCl in buffer A). For the purification of C-His-NP, the clarified lysate was mixed with Ni-NTA agarose (Qiagen) for 1 h, washed twice with buffer A (lysis buffer containing 20 mM imidazole), and eluted with buffer B (lysis buffer containing 250 mM imidazole). Fractions containing NP, as determined by Coomassie blue sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, were pooled and applied to a 5-ml HiTrap Heparin HP column (Amersham Biosciences), and NP was eluted at an NaCl concentration of 900 mM. The protein peak was collected and further purified by gel filtration chromatography on Superdex 200 and Superose 6 columns (Amersham Biosciences) in 50 mM Tris-HCl (pH 7.5) and 200 mM NaCl. Purified NP protein was concentrated to 5 mg/ml using a Centricon tube (Millipore) and stored at −80°C.

Assays for viral RNA transcription and replication in vitro. The 50-nt NS (genome segment 8) vRNA and cRNA templates were chemically synthesized and purified by Integrated DNA Technologies. The capped RNA primer was alfalfa mosaic virus (ALMV) RNA 3 containing an m^7GpppGm cap structure prepared as described previously (29). The 50-nt wild-type (wt) or U3A vRNA (40 pmol), without and with ALMV RNA 3, was incubated with the purified viral polymerase (0.3 μg, 1.2 pmol) for 15 min at room temperature in a final volume of 25 μl in reaction buffer [50 mM HEPES, pH 7.8; 5 mM $Mg(OAc)_2$; 2 mM dithiothreitol; 5 units RNase inhibitor). After addition of ATP, CTP, and GTP (each at 1 mM final concentration) and [α-^{32}P]UTP (0.01 mM, 3 Ci/mmol), the mixture was incubated for 1 h at 30°C. For cRNA synthesis assays, the polymerase complex (0.3 μg, 1.2 pmol) was preincubated with U3A vRNA (40 pmol) for 15 min at room temperature in a final volume of 25 μl in the reaction buffer. NP (0.3 μg, 5.4 pmol) was then added, where indicated. After addition of the four nucleoside triphosphates, the mixtures were incubated for 1 h at 30°C. For vRNA synthesis assays, the polymerase complex (1.2 pmol) was preincubated with the 50-nt NS cRNA (40 pmol) for 15 min at room temperature. NP (5.4 pmol) was then added, where indicated, and after addition of the four nucleoside triphosphates, the mixtures were incubated for 1 h at 30°C. Where indicated, NP was preincubated with 0.066 pmol of the ~3,000-nt-long single-stranded DNA (ssDNA) described below for 15 min at room temperature prior to its addition to the cRNA and vRNA replication assays. RNA was isolated by phenol-chloroform extraction and precipitated with isopropanol. RNA was resuspended in loading buffer (47.5% formamide, 0.25 mM EDTA, 0.0125% SDS), heated for 5 min at 95°C, and resolved on 10% polyacrylamide gels containing 8 M urea. Labeled RNA products were quantitated using the Bio-Rad PhosphoImager.

Gel shift assays. NP (0.3 μg, 5.4 pmol) was incubated for 20 min at room temperature with an ssDNA (0.06 μg, 0.066 pmol), ~3,000 nt long, which was generated using the pGEM-T Easy vector (Promega). The 50-nt NS vRNA (0.6 pmol), which was ^{32}P labeled at its 5′ end using T4 polynucleotide kinase, was then added, and the mixture was incubated for 1 h at 30°C in the reaction buffer used for cRNA and vRNA synthesis. NP-vRNA complexes and free vRNA were resolved on a nondenaturing 4% polyacrylamide gel in 45 mM Tris-borate, pH 8.3, 1 mM EDTA as running buffer.

Immunoprecipitation. NP (0.3 μg), which was preincubated for 20 min at room temperature with ssDNA (0.06 μg), was incubated in the presence or absence of the purified viral polymerase (3 μg) for 15 min at 30°C in the reaction buffer used for cRNA and vRNA synthesis. Anti-His antibody was then added, and the immunoprecipitation was carried out for 30 min at 30°C. Protein A agarose beads were added in the presence of 1% NP-40, 0.5% deoxycholate, 5 μg/ml aprotinin, and 5% bovine serum albumin (BSA), followed by incubation for 30 min at 4°C. The beads were washed at 4°C twice with a buffer containing 20 mM HEPES, 5 mM $Mg(OAc)_2$, 2 mM dithiothreitol, 1% NP-40, 0.5% deoxycholate, 5 μg/ml aprotinin, and 5% BSA and twice with the same buffer supplemented with 500 mM NaCl, The protein A agarose beads were eluted with the SDS gel loading buffer and separated on a 10% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane, which was probed with an antibody directed against the major structural proteins of influenza A/Udorn/72 virus (provided by Robert A. Lamb). This antibody detects only the NP and M (matrix) protein of the PR8 virus on immunoblots. After the mem-

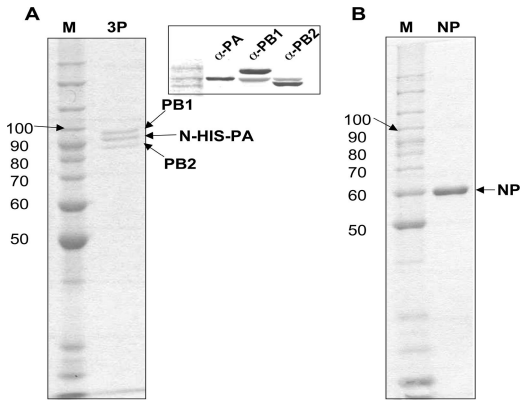

FIG. 1. Purity of preparations of purified influenza virus polymerase (A) and purified untagged NP protein (B). These two preparations were analyzed by electrophoresis on 9% SDS-polyacrylamide gels, followed by Coomassie blue staining. The positions of protein molecular mass markers (lanes M; kDa) are shown on the left of the two gels. Immunoblot assays were carried out to confirm the identity of the three polymerase proteins (shown in panel A), using polyclonal anti-PA antibody (provided by Krister Melen and Ilkka Julkunen) and anti-PB1 and PB2 antibodies (7, 41). A small amount of nonspecific sticking of the latter two antibodies to PA was observed.

brane was stripped, it was probed with anti-PA antibody (provided by Krister Melen and Ilkka Julkunen).

RESULTS

Purification of baculovirus-expressed influenza virus RNA-dependent RNA polymerase and nucleocapsid protein and choice of RNA templates. To isolate assembled influenza virus polymerase complexes, Sf21 insect cells were coinfected with three baculovirus vectors, one expressing the PA protein containing an N-terminal His tag and the other two expressing untagged PB1 and PB2 proteins. The viral polymerase complexes that assembled in the insect cells were purified using an Ni-NTA resin, followed by heparin affinity chromatography, gel filtration, and anion-exchange chromatography. As shown by a Coomassie blue-stained SDS-polyacrylamide gel, the purified viral polymerase complex (3P) contained equal amounts of the PA and PB1 proteins and a somewhat smaller amount of the PB2 protein (Fig. 1A). The identities of these proteins were verified by immunoblot assays. Some loss of the PB2 protein occurred during the purification procedure, indicating that its association with a polymerase complex purified via a tagged PA protein was not as strong as that of PB1. Usually the loss of the PB2 protein was relatively small, like that shown in Fig. 1A. In the rare instances when the loss of PB2 was much more substantial, the resulting polymerase complex had minimal or no activity in the transcription and RNA replication assays described below (data not shown), consistent with the observations of others that a tripartite polymerase complex is required for transcription and RNA replication (16–18, 20) (see Discussion). Untagged NP protein, which was expressed separately using a baculovirus vector, was purified using ammonium sulfate precipitation, heparin affinity chromatography, gel filtration, and cation-exchange chromatography, and the purified preparation was analyzed by gel electrophoresis (Fig. 1B). The NP protein is the only Coomassie blue-stained band detected in this gel analysis. Purified C-His-NP exhibited the same gel pattern (data not shown). If any contaminating insect cell or baculovirus proteins are present in the purified viral polymerase and/or NP protein preparations, their amounts are at levels not detectable by this analysis.

In the present study we used chemically synthesized RNA templates of 50 nt in length containing the 25 5′- and 3′-terminal nt of NS vRNA or of NS cRNA of influenza A/Udorn/72 virus. With these templates, only minimal amounts of smaller RNA products were synthesized in the in vitro influenza virus polymerase assays, as documented below.

The purified influenza virus complexes catalyze capped RNA-dependent transcription in vitro in the absence of the NP protein. As the first step in establishing an in vitro replication system, we determined whether the purified, baculovirus-expressed influenza virus polymerase complexes are active in transcription, i.e., the synthesis of capped RNA-primed, poly(A)-containing mRNA in the absence of NP. For these experiments, we used the capped RNA primer ALMV RNA3 containing an m^7GpppGm cap, which should be cleaved by the intrinsic cap-dependent endonuclease of the viral polymerase at a CA sequence that is 11 and 12 bases downstream from the cap (35). The resulting capped RNA fragment would serve as primer for the synthesis of an RNA terminating within the tract of six U bases located 29 to 34 bases from the 3′ end of the 50-nt vRNA template, and the resulting ~50-nt RNA would then be polyadenylated (15, 37). To determine whether such polyadenylated RNA was synthesized, the purified RNA polymerase was incubated with the 50-nt NS vRNA template in the absence or presence of capped ALMV RNA 3 for 15 min at room temperature, followed by a 1-h incubation at 30°C in the presence of ATP, CTP, GTP, and [α-^{32}P]UTP. As predicted, gel electrophoretic analysis of the capped RNA-primed RNA products showed a heterogeneous set of RNAs ranging in length from approximately 50 nt to significantly more than 100 nt (Fig. 2A, lanes 2 and 3), which were selected on an oligo(dT) resin (Fig. 2B), verifying that they are polyadenylated transcripts. It has been shown previously that the influenza virus polymerase in vitro adds large poly(A) chains, 175 to 350 nt in length (30). We presume that the RNA products containing the longest poly(A) chains (i.e., RNAs longer than 100 nt) were not efficiently eluted from the oligo(dT) resin.

In addition, the transcription products contained a discrete species of approximately 60 nt, representing approximately 20% of the total product, as determined by phosphorimager analysis (Fig. 2A, lanes 2 and 3). The heterogeneous polyadenylated RNAs comprised the majority (~80%) of the product RNAs. The identity of the 60-nt product was established by carrying out a reaction with a 50-nt vRNA template containing a disrupted U tract (3′-UUUAU-5′) at positions 29 to 34, designated as the U3A vRNA template. This disruption of the U tract has been shown to eliminate termination and poly(A) addition in the absence of NP (32). This was shown to be the case: little or none of the heterogeneous, polyadenylated RNAs of large size was synthesized, and the predominant product was the 60-nt RNA (Fig. 2A, lane 4), which corresponds to the full-length transcript that fails to terminate at the disrupted U tract. The presence of some 60-nt RNA in the wt vRNA reaction (lanes 2 and 3) indicates that some polymerase molecules fail to terminate at the intact U tract in the absence

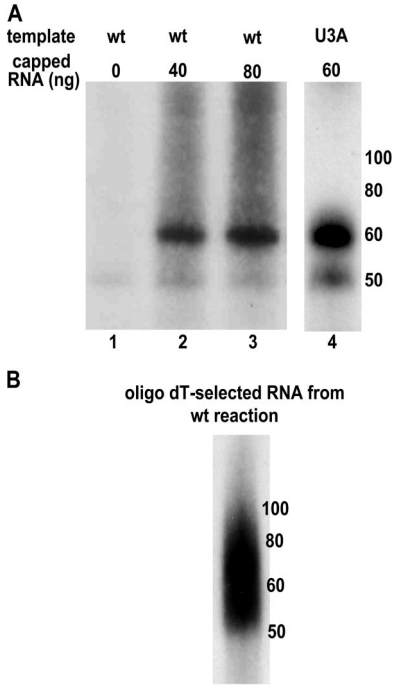

FIG. 2. The purified influenza virus polymerase is active in capped RNA-primed transcription in vitro. (A) Transcription was carried out using either the wt 50-nt vRNA template (lanes 1 to 3) or the U3A vRNA template (lane 4) and with the indicated amounts of the ALMV RNA 3 capped RNA primer. The RNA products were analyzed by electrophoresis on a 10% 8 M urea-polyacrylamide gel. The positions of RNA markers with the indicated number of nucleotides are shown on the right. (B) RNA synthesized in the presence of ALMV RNA 3 capped RNA (80 ng) was selected on an oligo(dT) resin and analyzed by electrophoresis on a 10% 8 M urea-polyacrylamide gel.

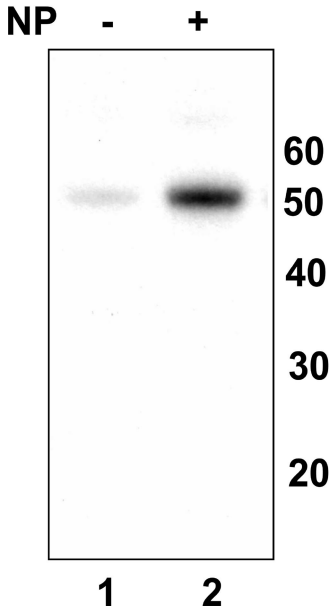

FIG. 3. NP strongly stimulates unprimed cRNA synthesis by the viral polymerase. The viral polymerase was incubated with the U3A vRNA template in the absence (−, lane 1) or in the presence (+, lane 2) of 5.4 pmol of untagged NP protein, and the RNA products were analyzed on a 10% 8 M urea-polyacrylamide gel. The positions of RNA markers with the indicated number of nucleotides are shown on the right.

of NP, which has not been observed in transcription reactions catalyzed by the viral polymerase that is associated with the viral RNPs that are formed in infected cells (2) (see Discussion).

The NP protein potentiates the synthesis of cRNA, the first step in viral RNA replication, in the absence of a primer. Our main goal was to establish an in vitro system that catalyzes unprimed cRNA and vRNA synthesis using purified recombinant proteins. For these experiments we used purified NP that was either untagged or contained a C-terminal His tag; the same results were obtained with the two NP preparations. For cRNA synthesis, we used the U3A vRNA template, thereby eliminating termination and poly(A) addition in the absence of the NP protein (32) (Fig. 2A). Consequently, we could focus solely on functions of the NP protein in initiating cRNA synthesis rather than its role in antitermination at the U tract. The 50-nt U3A vRNA was first incubated with the purified viral polymerase to enable its 3′ end to bind to the PB1 subunit of the polymerase (18). This was then followed by the addition of purified NP protein, which binds to the rest of the vRNA template as well as carrying out any other function needed for unprimed cRNA synthesis. It was necessary to follow this procedure to coat the vRNA template with the NP protein because of its ability to nonspecifically bind any RNA at 24-nt intervals from one end to the other (1, 14, 46, 47). Consequently, as expected, addition of the NP protein to the U3A vRNA prior to the viral polymerase blocked its ability to interact with the viral polymerase, presumably because the 3′ end of the vRNA was occupied by the NP protein. For the control reaction, the NP protein was not added after the vRNA-polymerase preincubation. The reaction mixtures were incubated for 1 h at 30°C in the presence of ATP, CTP, GTP, and [α-^{32}P]UTP. Gel electrophoretic analysis of the RNA products showed that NP (5.4 pmol) strongly stimulated the synthesis of a 50-nt-long cRNA (Fig. 3). Maximum stimulation occurred with this amount of NP. A small amount of synthesis of a 50-nt cRNA was consistently detected in the absence of NP (lane 1), ranging in various experiments from 2 to 5% of that synthesized in the presence of NP (lane 2). Small amounts of this 50-nt cRNA were also synthesized in the absence of NP in the transcription reactions of Fig. 2. No cRNA product was detected in the absence of the template vRNA or of the viral polymerase (data not shown).

The RNA-binding activity of the NP protein is not required for stimulation of unprimed synthesis of a 50-nt cRNA. To distinguish between the three models for the function of the NP protein in the switch to unprimed synthesis of cRNA, we determined whether NP lacking RNA-binding activity retains the ability to strongly stimulate cRNA synthesis. Two of the models attribute this function of NP to its RNA-binding activity (see the introduction). The X-ray crystal structure of the NP protein reveals an RNA-binding groove that contains multiple positively charged amino acids widely distributed in the NP primary sequence (47). We have not yet succeeded in completely inactivating NP RNA binding by mutation of several groups of these charged amino acids, possibly because several modes of RNA binding are possible. As an alternative, based

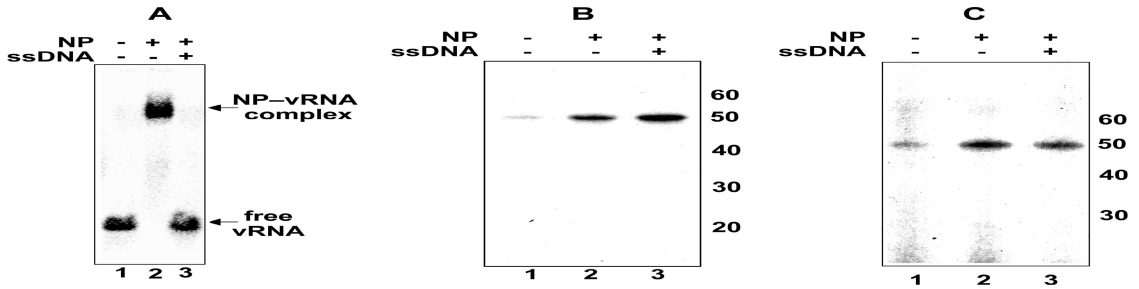

FIG. 4. The RNA-binding activity of the NP protein is not required for its stimulation of the unprimed synthesis of either a 50-nt cRNA or a 50-nt vRNA. (A) Gel shift assay demonstrating that saturation of the RNA-binding domain blocks the subsequent binding of vRNA. C-His-NP was preincubated with ssDNA, followed by incubation with 5′-^{32}P-labeled 50-nt NS vRNA (lane 3). Lane 1, vRNA alone. Lane 2, vRNA incubated with NP. NP-vRNA complexes were resolved from free vRNA by nondenaturing gel electrophoresis. (B) NP lacking RNA-binding activity efficiently stimulates the synthesis of a 50-nt cRNA. The viral polymerase was preincubated with the 50-nt vRNA template, followed by the addition of either C-His-NP (lane 2) or C-His-NP saturated with ssDNA (lane 3). Lane 1, no addition of C-His-NP. The RNA products were analyzed on an 8 M urea-10% polyacrylamide gel. Phosphorimager analysis of five separate assays showed that the amount of the 50-nt cRNA product synthesized in lane 3 was 90 to 110% of that synthesized in lane 2. The positions of RNA markers with the indicated numbers of nucleotides are shown on the right. (C) NP lacking RNA-binding activity efficiently stimulates the unprimed synthesis of a 50-nt vRNA. The viral polymerase was preincubated with the 50-nt cRNA template, followed by the addition of either C-His-NP (lane 2) or C-His-NP saturated with ssDNA (lane 3). Lane 1, no addition of C-His-NP. Phosphorimager analysis of five separate assays showed that the amount of the 50-nt vRNA product synthesized in lane 3 was 90 to 110% of that synthesized in lane 2. The positions of RNA markers with the indicated numbers of nucleotides are shown on the right.

on the fact that NP binds RNA nonspecifically (1, 14, 46, 47), we determined whether saturating the NP protein with ssDNA would block the subsequent binding of single-stranded RNA, including vRNA, thereby providing us with NP proteins lacking RNA-binding activity. As shown in Fig. 4A (lanes 1 and 2), incubation of NP (5.4 pmol) with 5′-^{32}P-labeled NS U3A vRNA (0.6 pmol) bound essentially all the vRNA and shifted it into an NP-vRNA complex. When NP was preincubated with 0.066 pmol of a ~3,000-nt-long ssDNA for 15 min at room temperature, a subsequent incubation with vRNA for 1 h did not produce an NP-vRNA complex (lane 3), demonstrating that NP presaturated with ssDNA no longer bound vRNA. The amount of ssDNA used in this experiment corresponds to approximately 30 nt per NP protein molecule, exceeding the 24 nt of RNA per NP protein molecule found in viral nucleocapsids (4, 28). No detectable vRNA binding to the presaturated NP occurred even when the vRNA level was increased more than 25-fold (data not shown), showing that the ssDNA could not be displaced from NP by higher levels of vRNA.

We used this saturating level of ssDNA to block the RNA-binding activity of NP during unprimed cRNA synthesis in vitro. As shown in Fig. 4B (lanes 2 and 3), NP lacking RNA-binding activity retained essentially all its ability to strongly stimulate the synthesis of a 50-nt cRNA product. We conclude that the RNA-binding activity of NP is not required for its activity in stimulating unprimed cRNA synthesis catalyzed by the viral polymerase. In addition, because the vRNA template in the reaction mixture containing the ssDNA-saturated NP (lane 3) is not coated with NP, the synthesis of a 50-nt cRNA does not require that the 50-nt vRNA template be associated with NP.

NP lacking RNA-binding activity stimulates unprimed synthesis of a 50-nt vRNA. The second step in viral RNA replication is the synthesis of vRNA using cRNA as a template. To determine whether the RNA-binding activity of the NP protein is required for vRNA synthesis, the viral polymerase was incubated in the absence and presence of NP that was presaturated with ssDNA. As shown in Fig. 4C (lanes 2 and 3), NP lacking RNA-binding activity retained all of its capacity to strongly stimulate the synthesis of a 50-nt vRNA product. The results in lane 3 also show that the synthesis of a 50-nt vRNA product does not require that the 50-nt cRNA template be associated with NP. A small amount of the synthesis of a 50-nt vRNA was consistently detected in the absence of NP (lane 1), approximately 2 to 5% of that synthesized in its presence.

NP lacking RNA-binding activity binds to the viral polymerase. The third model for the function of the NP protein in the switch to unprimed synthesis of cRNA requires that NP bind directly to the viral polymerase (see the introduction). To determine whether NP lacking RNA-binding activity binds to the viral polymerase, NP was preincubated with ssDNA and then added to the purified viral polymerase, in which the PA subunit contained an N-terminal His tag. The control sample lacked the viral polymerase. These mixtures were incubated for 15 min at 30°C under the conditions used for cRNA synthesis and were then immunoprecipitated with anti-His antibody (Fig. 5). An immunoblot with an anti-NP antibody showed that NP coimmunoprecipitated with the viral polymerase. A small amount of NP nonspecifically bound to the protein A-Sepha-

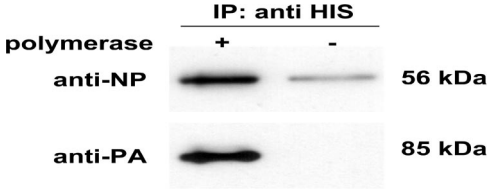

FIG. 5. NP lacking RNA-binding activity binds to the viral polymerase. Untagged NP presaturated with ssDNA was incubated with the viral polymerase containing PA with an N-terminal His tag (+ lane) or with buffer (− lane), and the polymerase was immunoprecipitated with anti-His antibody. The immunoprecipitates were immunoblotted with antibody that detects the 56-kDa NP protein. After the immunoblots were stripped, they were probed with anti-PA antibody.

rose resin in the absence of the viral polymerase, as also found by others (17). An immunoblot assay with an anti-PA antibody verified that PA was in the immunoprecipitate. The same results were obtained when the immunoprecipitation was carried out in the presence of an excess (2.5 μg) of BSA. We conclude that NP lacking RNA-binding activity binds to the viral polymerase and that this binding does not require the presence of the vRNA template.

DISCUSSION

We have used purified recombinant influenza virus polymerase complex and NP protein to establish an in vitro system that catalyzes the two steps of influenza virus RNA replication, the synthesis of cRNA and vRNA in the absence of a primer. The template RNAs were 50-nt-long vRNA and cRNA molecules, and the RNA products consisted almost entirely of full-length 50-nt-long cRNA and vRNA chains, respectively, with little or none of smaller species. These cRNA and vRNA products presumably initiate with a ribonucleoside triphosphate, as shown by other studies of cRNA and vRNA synthesis (6). Because the purified polymerase and NP protein preparations used in our assays were obtained from insect cells and contain no major contaminating proteins, our results suggest that the initiation of unprimed RNA synthesis does not require previously reported mammalian host factors (12, 13, 21, 23, 24, 26, 39). Nonetheless, our results do not rule out the possibility that such mammalian host factors might regulate or further enhance viral RNA replication in infected cells. In fact, our purified in vitro system makes it possible to accurately assess the functional roles of such host factors, which can be separately expressed, purified, and added to our in vitro assays.

We used this purified in vitro replication system to evaluate the three models for the role of NP in the switch from capped RNA-primed transcription to unprimed viral RNA replication. Two models attribute this function of NP to its RNA-binding activity, either by binding to the template RNA or by binding to nascent cRNA and vRNA chains (33, 43). Our results rule out these two models, because we demonstrated that NP RNA-binding activity is not required for the stimulation of unprimed synthesis of 50-nt cRNA and vRNA. We blocked the RNA-binding site(s) of NP using ssDNA and showed that such NP proteins retained their ability to stimulate the synthesis of these 50-nt cRNA and vRNA molecules. The third model, the polymerase modification model, proposes a direct protein-protein interaction between NP and the viral polymerase, resulting in a modification of the polymerase in favor of unprimed initiation (33). Our results support this model. We showed that the NP protein that lacks RNA-binding activity binds directly to the viral polymerase: NP was coimmunoprecipitated using anti-His antibody directed against the N-terminal His tag of the PA subunit of the viral polymerase.

Previous results in favor of the polymerase modification model included coimmunoprecipitation experiments in cell extracts, either from virus-infected cells or from cells cotransfected with plasmids expressing NP and the three P proteins (3, 17). In an early study NP was found to coimmunoprecipitate with two polymerase subunits, PB1 and PB2, but PA was not detected in these immunoprecipitates (3). In contrast, a recent study found that NP coimmunoprecipitated all three polymerase proteins and reported that the level of viral RNA synthesis correlated with the efficiency of NP protein binding to the trimeric viral polymerase (17). Because we used an antibody directed against PA to coimmunoprecipitate NP, we verified that PA is part of the polymerase-NP complex. Further, by using purified polymerase and NP proteins, we showed that the interaction of NP with the polymerase is most likely direct and is not mediated by cellular proteins or other viral proteins.

The mechanism by which NP binding to the viral polymerase potentiates unprimed viral RNA replication has not been determined. The most likely hypothesis is that the binding of NP to one or more of the polymerase proteins leads to a conformational change in the polymerase complex that activates unprimed initiation of synthesis of cRNA and vRNA. Such an effect of the influenza virus NP protein is comparable to the situation with vesicular stomatitis virus, where the association of the viral nucleocapsid protein with the dimeric viral polymerase transforms the viral polymerase from a transcriptase into a replicase (34). Interestingly, we consistently detected a low level of unprimed synthesis of 50-nt-long cRNA and vRNA chains in the absence of NP, indicating that a small number of influenza virus polymerase complexes apparently have acquired such a conformational change in the absence of NP. It is not known which polymerase protein(s) in trimeric polymerase complexes interacts with NP, thereby resulting in the activation of unprimed cRNA and vRNA synthesis. Previous experiments have not addressed this issue but instead have assayed the binding of NP to individual polymerase proteins that are not in functional polymerase complexes. For example, transfection experiments in which NP was expressed with individual polymerase subunits found that NP coimmunoprecipitated with PB1 and PB2 (3). Although coimmunoprecipitation of PA with NP was not detected, it is not clear whether this result was due to a lack of interaction or to the extremely low expression of PA in these experiments.

After activation by NP binding, all three polymerase protein subunits most likely have roles in unprimed viral RNA replication. The PA subunit undoubtedly has a role because a virus containing a mutation in the PA gene exhibits a temperature-sensitive phenotype in viral RNA replication (16). A genetic linkage has also been observed between PB2 and NP (20), suggesting a role for PB2 in NP-stimulated viral RNA replication. In addition, recent transfection experiments showed that PB2 as well as PA has a significant role in NP-stimulated viral RNA synthesis and provided evidence that the role of PB2 in this process was enhanced by the presence of K rather than E at position 627 (17). The influenza A/PR/8/34 PB2 virus protein used in the present study has K at 627, and future studies will determine the effect of substituting E at this position in PB2 on NP-stimulated unprimed RNA replication in vitro. The PB1 subunit provides both the catalytic site for nucleotide addition and the binding site for the 3′ end of the vRNA and cRNA templates (18). Our goal is to utilize the purified in vitro viral RNA replication system to elucidate the molecular mechanisms of NP-stimulated unprimed synthesis of cRNA and vRNA.

Our results may also provide insights into the role(s) of the NP protein molecules that coat the vRNA and cRNA templates at 24-nt intervals when transcription and replication occur in infected cells. One function has been ruled out: these

bound NP protein molecules do not protect vRNA against RNase digestion (1, 8, 31), which is also presumably the case for cRNA. Our investigation of the role of the NP bound to vRNA or cRNA templates was limited by the fact that it is not feasible to coat these templates with NP prior to incubating them with the polymerase, because such NP precoating blocks the 3′ end of these RNA templates from binding to the polymerase. For replication reactions, the vRNA and cRNA template that was first bound to the polymerase could be subsequently coated with NP because NP is in fact required for directing the polymerase toward unprimed initiation of viral RNA replication. Using this procedure, we found that NP coating of a 50-nt vRNA and cRNA template was not required for their activity in replication in vitro, in that ssDNA-saturated NP stimulated replication of these two 50-nt RNA templates as efficiently as untreated NP. It will be of interest to determine whether NP binding to longer vRNA and cRNA templates facilitates their copying, possibly by melting RNA secondary structures (1) that would otherwise cause premature termination of synthesis.

For transcription reactions, the vRNA template that was bound to the polymerase could not be subsequently coated with NP because the addition of NP directs a substantial proportion of the polymerases toward unprimed initiation of viral RNA replication (unpublished experiments). Using this naked vRNA template, we obtained an unanticipated result, namely, that some polymerase molecules failed to terminate at the intact U tract located 29 to 34 bases from the 3′ end of the 50-nt vRNA template. In contrast, such a failure to terminate was not observed in transcription reactions catalyzed by the viral polymerase that is associated with the viral RNPs that are formed in infected cells (2). These results suggest that the absence of NP proteins on the vRNA template that was used in the present study is responsible for the failure of some polymerase complexes to terminate at the wt U tract. One possibility is that the rate of transcription on a naked vRNA template is higher than that on a vRNA template coated with NP at 24-nt intervals, because in the latter case the polymerase needs to displace multiple NP proteins to copy the vRNA. As a consequence, some polymerase molecules rapidly traverse the intact U tract on a naked vRNA template and are not delayed long enough to reiteratively copy the U tract. This hypothesis cannot be tested at present because it is not feasible to use a vRNA template that is coated with NP protein molecules for transcription reactions. A more general statement of this hypothesis is that vRNA-bound (and cRNA-bound) NP protein has two roles: (i) to slow down the polymerase to reduce errors in copying these templates and (ii) to melt secondary structures in the template RNA that would otherwise lead to premature termination of synthesis.

The X-ray crystal structure of the influenza A virus NP protein shows that it folds into a two-domain structure, with the two domains designated as the head and body domains (47). This structure identified the RNA-binding domain of NP: a large positively charged groove at the exterior of the molecule at the interface between the two domains. Because such a large number of positive amino acids (R and K) are present in this groove, it has been difficult to identify the positive amino acids that are absolutely required for RNA binding. We are continuing these studies, particularly because a mutant NP protein lacking RNA-binding activity is important for the elucidation of the role of NP in viral RNA replication. Such a mutant NP protein would enable us not only to verify that the RNA-binding activity of NP is not required for the stimulation of unprimed initiation of cRNA and vRNA synthesis but also to determine whether binding of a single-stranded nucleic acid, either ssDNA or single-stranded RNA, to the NP protein is required for the NP protein to acquire the ability to direct the polymerase to unprimed initiation of viral RNA replication. The structural study of the NP protein also showed that a short tail loop mediates NP-NP oligomerization (47). However, it has not been determined which NP amino acid region mediates the interaction with the trimeric viral RNA polymerase that directs it toward unprimed viral RNA replication. Transfection assays have shown that mutation of four NP amino acids results in reduced viral RNA replication (22). These amino acids are located in the body domain of NP (47), suggesting that the site(s) that functionally interacts with the trimeric viral polymerase is in this domain. It should now be possible to identify the specific region(s) of NP that functionally interacts with the trimeric viral polymerase using our in vitro RNA replication system.

ACKNOWLEDGMENTS

This investigation was supported by NIH grants AI11772 (R.M.K.) and AI067638 (Y.J.T.), Welch grant C-1565 (Y.J.T.), and NIH NRSA fellowship F32 A1058581 (L.L.N.).

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